Effects of Coal Combustion Products and Metal Compounds on Sister Chromatid Exchange (SCE) in a Macrophagelike Cell Line

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Investigations of genotoxic effects of particles have almost exclusively been performed by organic extraction, while direct investigations in cells capable of engulfing particles have only been performed in few cases. Thus, in most studies, the eventual effects of particle-associated metal compounds have remained undiscovered. The present study attempted direct measurement of genotoxic effects of particulate coal combustion products by using the P388D$_1$ macrophage cell line. The capability of these cells for phagocytosis was demonstrated with insoluble particles. The sister chromatid exchange (SCE) test was used for measuring genotoxic effects of test compounds.

Dimethylnitrosamine and benzo(a)pyrene did not increase SCE, indicating that the P388D$_1$ cell line has lost the capacity for metabolism of latent organic carcinogens, reducing the value of these cells for evaluating genotoxic effects of complex particles. Indirect evidence has been obtained that the cell line may be infected with a virus. Thus, interactions between virus and test compound may lead to erroneous results. This should be kept in mind during evaluation of the results.

The effects of metals with reported carcinogenic or mutagenic effects on SCE were compared in P388D$_1$ cells and human lymphocytes: NaAsO$_2$, CdCl$_2$, K$_2$Cr$_2$O$_7$, CoCl$_2$, CH$_3$HgCl and MnSO$_4$ increased SCE in both cell systems. Pb(CH$_3$COO)$_2$, BeSO$_4$ and NiSO$_4$ had a weak effect on SCE in P388D$_1$. Pb(CH$_3$COO)$_2$ and NiSO$_4$, but not BeSO$_4$, increased SCE in human lymphocytes. Cr(CH$_3$COO)$_3$ increased SCE in human lymphocytes at high concentration, but was a strong inducer of increased SCE in P388D$_1$ cells, which take up Cr(III) by phagocytosis. This suggests that the Cr(III) ion is an ultimate carcinogenic form of chromium. Generally P388D$_1$ cells and human lymphocytes respond to *in vitro* exposure to metals in agreement with reported mutagenic/carcinogenic effects of the metals.

Of four precipitated coal fly ash samples tested, only one sample (from an electrostatic precipitator downstream of a cyclone at an electricity generating plant burning pulverized coal) had a clear increasing effect on SCE in P388D$_1$. Extraction of this sample with medium with 10% serum yielded an extract capable of increasing SCE. The extracted particles still increased SCE, but less than unextracted particles.

Emission samples taken with impactor sampler after a dilution probe using controlled cooling of the flue gas were obtained from an oil-fired (one sample) and a coal-fired (one sample < 3 μm and one sample < 3 μm) steam boiler. The only sample increasing SCE was the sample < 3 μm from the coal-fired boiler. Extract from this sample also increased SCE.

The results demonstrated that the use of phagocytizing cells for assessment of genotoxic effects of particles offers a useful system, since the particles can be investigated directly. Furthermore, particles, extracted particles and extract can be investigated in the same system.

Introduction

Cytotoxicity tests of particles related to air pollution have been performed directly in macro-

phages (1-3). In contrast, investigations of the mutagenicity of particulate air pollutants have almost exclusively used extraction of particles with organic solvents followed by test of the extracts in the Salmonella assay (4-6). Extracts are also used in most correlation studies (7,8).

In such studies mutagenic or interacting effects of inorganic compounds in the particles will remain

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undiscovered. So far, there is a lack of knowledge about the importance of inorganic components in air pollution-related particles with regard to mutagenic properties. Direct assessment *in vitro* of the mutagenicity of whole particles has only been tried in a few studies, e.g., in the protozoan *Paramecium tetraaurelia* (*9,10*). These studies did indicate the presence of inorganic mutagens in coal fly ash.

Development of mammalian cell-based assays testing particles directly would be useful for assessment of the relevance of a high mutagenicity in the Salmonella assay of organic extracts from air pollution particulate samples in relation to the lung carcinogenicity in humans of such particles. The most relevant way to investigate the bioavailability of particle-associated mutagens is the use of macrophages, since after lung deposition, particles, e.g., from diesel emissions, are rapidly cleared by pulmonary alveolar macrophages (PAM) (*11*).

In most mammalian cell-based *in vitro* systems presently in use, the mutagenicity of complex particles cannot be determined directly because insoluble or slightly soluble components of the test material reside outside the cells. This has been circumvented in the present study by use of a continuous macrophage cell line, P388D$_1$, generously given to me by Clyde Dawe, Department of Pathology, NCI. This cell line originates from a methylcholanthrene-induced lymphoid neoplasm (P388) of a DBA/2 mouse (*12*). After 49 serial mouse passages, the P388 cells spontaneously underwent morphological alteration *in vitro* from lymphoid to amoeboid cell type, and after IP injection of these altered cells, the resulting ascites tumor was also of altered morphology. From these cells the P388D$_1$ ("derived") line was initiated. After 154 passages *in vitro* the P388D$_1$ cell line was still malignant in DBA/2 mice. Koren et al. (*13*) demonstrated that P388D$_1$ cells morphologically and functionally are macrophages: They phagocytize latex particles and adhere firmly to glass and plastic. They carry receptors for complement (C3) and immunoglobulin (Fc), but lack surface Ig and do not bind fluorescent rabbit anti-mouse brain serum (a T-cell marker). P388D$_1$ cells act as killer cells in an antibody-dependent cell-mediated cytotoxicity system. Wade et al. (*14*) used the P388D$_1$ cell line to evaluate the toxicity of asbestos fibers *in vitro*.

The present study investigated whether the P388D$_1$ cell line is suited for testing the genotoxicity of slightly soluble, complex particles present in environmental pollution. The ability of the cell line for phagocytosis has been demonstrated with Cr_2O_3 and different types of fly ash. The SCE test was used for evaluation of genotoxicity, since it gives results in good agreement with mutagenic and/or

carcinogenic properties of inorganic and organic compounds after *in vivo* or *in vitro* exposure: Smoking (*15,16*), occupational exposure to vinyl chloride (*17*), lead (*18*) and organic solvents (*19*) and medication with arsenic (*20*) have been reported to induce elevated SCE in peripheral lymphocytes from exposed humans. *In vitro* exposure of mammalian cells to organic carcinogens increase SCE (*21-25*). Andersen et al. (*26*) found that five metal compounds with reported mutagenic or carcinogenic effects increased SCE in human lymphocytes, but not in Chinese hamster V79-E cells. This study demonstrates that compounds of most carcinogenic metals increase SCE in P388D$_1$ cells and human lymphocytes.

The results reported here describe effects of *in vitro* exposure to organic latent carcinogens and different types of fly ash from precipitators of coal-fired power plants on SCE in P388D$_1$. Emission samples collected at an oil- and a coal-fired plant have also been tested for effect on SCE in P388D$_1$ cells. Although the results show that the P388D$_1$ cell line does not metabolize and activate latent organic carcinogens, they demonstrate that genotoxic effects of pure inorganic compounds and complex particles can be measured in this cell line.

During the course of the experiments, indirect evidence has been obtained that the P388D$_1$ cell line is infected with a virus. The possibility for interacting effects between virus and carcinogens, which has been demonstrated to take place in the simian adenovirus hamster embryo cell (SA7/HEC) system, both with metals (*27*) and organic carcinogens, (*28*) should be kept in mind during evaluation of the results.

The results may be affected in two ways: (1) viral-induced toxicity may lower the response to SCE-inducing agents (false negatives); (2) synergism between virus and tested agents may lead to overestimation of SCE-inducing capacity (false positives).

Materials and Methods

Human peripheral blood was obtained from randomly selected donors. P388D$_1$ cell cultures and human lymphocyte cultures were grown in Eagle's MEM with 50 µg/mL gentamycin and 10% fetal calf serum and 5-bromo-2'-deoxyuridine (BrdU) at 37.5°C in a 5% CO_2 atmosphere. Human lymphocyte cultures (0.5 mL blood in 10.0 mL final volume) also contained phytohemagglutinin (0.2 mL) and 5 i.e./mL heparin. The SCE assay was standardized for the P388D$_1$ cell line: 1-2 × 10^6 P388D$_1$ cells were exposed to 5 µg/mL BrdU and test compound for 48 hr. Human lymphocytes were exposed to BrdU for

72 hr (concentrations stated in the tables) and test compound for 24, 48 or 72 hr (stated in the tables).

Precipitated fly ash samples were collected from the flue gas cleaning devices of coal-fired plants. Emission samples from a coal-fired and an oil-fired plant were collected by a dilution probe-impactor sampler constructed at the Institute for Water and Air Quality Research, Göteborg, Sweden. The sampler, described elsewhere in detail (*29*), consists of a probe partially made of porous metal through which the flue gas is diluted with predried and preheated air to mimic the conditions in the plume with regard to condensation of gaseous components around particles, followed by a high volume cascade impactor separating the particles according to size. The plants and samples are briefly described in Table 1.

Metal compounds, thio-TEPA and dimethylnitrosamine (DMN) were solubilized in distilled water and sterile filtered immediately before addition to cultures. Fly ash, emission samples and Cr_2O_3 were suspended in water or medium as stated below. Additions to cultures were 1/10 of the final volume or less, and control cultures always received the same amount of water as the rest of the cultures. Benzo(a)pyrene (BP) was solubilized in dimethyl sulfoxide (DMSO), and control cultures received the same amount of DMSO as cultures exposed for BP. Colchicine was added to all cultures 2 hr before harvest. $P388D_1$ cells were harvested with a rubber policeman. After hypotonic treatment with $0.075M$ KCl for 10 min at 37.5° C, fixation with acetic acid/methanol (1:3) and production of chromosome slides, differential chromatid staining was performed as described in 15 or 30. After drying, the slides were mounted in Eukitt.

In experiments with human lymphocytes, 30 randomly selected metaphases with 46 chromosomes were counted from each culture. In experiments with $P388D_1$ cells 100 marker chromosomes were counted from randomly selected metaphases, since the caryotype contains 68 chromosomes, many of which are small acrocentrics. Two of the chromosomes, are easily recognizable marker chromosomes, large submetacentrics with a secondary constriction on the long arm. Samples were compared by using Student's *t*-test. The significance border was $p = 0.05$.

Results and Discussion

Effects of Pure Metal Compounds on SCE

The effects of compounds of nine metals on SCE in two cell systems have been investigated. The results are summarized in Tables 3-10. The metals have in common the fact that their compounds are known or suspected human carcinogens, have induced tumors in experimental animals or have been reported to have mutagenic effects *in vivo*.

Toxicity. In most experiments, concentrations ranging from "lethal" to "no effect" were used with 10 × dilution steps. Thus, the upper threshold concentrations stated in Table 2 are low approximations, but toxicity was observed at these concentrations. Since the standard SCE assay gives optimal frequency of differentially stained metaphases (i.e., second mitoses after addition of BrdU) in the control cultures at the time of harvest, toxicity can be seen as a decrease in second mitoses relative to first mitoses. Many metals are spindle inhibitors (*31*), and this effect will also reduce the number of second mitoses relative to first mitoses. Table 2 refers to experiments with BrdU present, and toxicity determinations without BrdU may give higher threshold concentrations. The same threshold concentrations for metal compounds were found in the two cell types, except after exposure to chromium compounds, most likely because reduction of Cr(VI) by erythrocyte glutathione (GSH) protects the lym-

Table 1. Characteristics of plants and samples.

Plant and sample code	Type of plant	Fuel combustion mode	Flue gas cleaning	Sample
A	Electricity generation	Pulverized coal	Electrostatic precipitator	Precipitated fly ash
B-1	Electricity generation	Pulverized coal	(1) Cyclone (2) Electrostatic precipitator	Precipitated fly ash from the electrostatic precipitator
B-2	Electricity generation	Pulverized coal	(1) Cyclone (2) Electrostatic precipitator	Flue gas desulfurization product
C	Electricity generation	Roast furnace	Cyclone	Precipitated fly ash
D-1	Steam boiler	Coal, moving grate	Many small cyclones	Precipitated fly ash
D-2	Steam boiler	Coal, moving grate	Many small cyclones	Emission sample < 3 μm
D-3	Steam boiler	Coal, moving grate	Many small cyclones	Emission sample > 3 μm
E	Steam boiler	Oil	Without flue gas cleaning	Emission sample

Table 2. Approximate highest concentrations of metal compounds in the growth medium allowing SCE determination after standard growth conditions (described in "Materials and Methods").

Metal compound	Highest concentration, M	
	P388D$_1$ cell line	HL[a]
NaAsO$_2$	2×10^{-6}	10^{-6}
NaAsO$_2$(CH$_3$)$_2$	10^{-5}	10^{-5}
Na$_2$HAsO$_4$	10^{-5}	nd[b]
BeSO$_4$	10^{-4}	10^{-4}
CdCl$_2$	10^{-5}	10^{-5}
CoCl$_2$	10^{-4}	10^{-5}
K$_2$Cr$_2$O$_7$	10^{-6}	10^{-5}
Cr(CH$_3$COO)$_3$	10^{-4}	10^{-3}
HgCl$_2$	10^{-5}	10^{-5}
CH$_3$HgCl	10^{-5}	10^{-5}
MnSO$_4$	10^{-5}	10^{-5}
NiSO$_4$	10^{-4}	10^{-5}
Pb(CH$_3$COO)$_2$	10^{-4}	10^{-4}

[a]Human lymphocytes.
[b]Not determined.

phocytes, and because phagocytic uptake increases the sensitivity of P388D$_1$ cells to Cr(III), and after exposure to CoCl$_2$ and NiSO$_4$.

Arsenic. Exposure to arsenite for 48 hr increased SCE in human lymphocytes from three donors (Table 3). Exposure to arsenite for 72 hr also increased SCE, while exposure for 24 hr had a smaller effect on SCE (data not shown). In P388D$_1$ cells, arsenite exposure had a marginal effect on SCE in two experiments. Other arsenic compounds tested (cacodylic acid and arsenate) did not increase SCE.

Arsenic medication was suggested to cause skin

Table 3. Effect of arsenic compounds on SCE.

Cell type	Compound	M	Increase in SCE compared with control, %	p[a]
P388D$_1$	NaAsO$_2$	10^{-6}	19	ns
–	–	10^{-7}	3.6	ns
–	–	10^{-8}	7.1	ns
HL[b,c]	–	10^{-6}	30	< 0.025
HL[b,d]	–	10^{-6}	117	< 0.0005
HL[b,d]	–	10^{-6}	40	< 0.0005
P388D$_1$	NaAsO$_2$(CH$_3$)$_2$	10^{-5}	–3.6	ns
–	–	10^{-6}	3.6	ns
HL[b,e]	–	10^{-5}	4	ns
–	–	10^{-6}	7	ns
P388D$_1$	Na$_2$HAsO$_4$	10^{-5}	2.7	ns
–	–	10^{-6}	8	ns
–	–	10^{-7}	0.9	ns

[a]ns = not significant.
[b]Four different donors were used; 48-hr exposure.
[c]20 µg/mL BrdU.
[d]10 µg/mL BrdU.
[e]4 µg/mL BrdU.

cancer in humans almost a century ago (*32*). Since then numerous published case reports and epidemiological studies concerning medical and occupational exposure as well as exposure through drinking water have furnished ample evidence that arsenic is a human carcinogen (*33*).

Inorganic and organic arsenic compounds have been tested for carcinogenicity in experimental animals by various routes of exposure in a number of studies, and negative results were obtained (*33*), except in two studies: subcutaneous administration of sodium arsenate to pregnant mice increased the incidence of lymphomas and lymphocytic leukemia in both the females and the offspring (*34*). Nine of fifteen rats surviving intratracheal instillation of a wineyard spray (Bordeaux mixture) containing calcium arsenate, copper sulfate and calcium hydroxide developed malignant lung tumors (*35*).

The mutagenicity of arsenic compounds in bacteria has been investigated in different systems: NaAsO$_2$, AsCl$_3$ and Na$_2$HAsO$_4$ were positive in the *rec*-assay in *B. subtilis*. In a tryptophan reversion assay with *E. coli*, rec A$^+$ but not rec A$^-$ strains were mutated by NaAsO$_2$ (*36*). These findings were confirmed by Kanematsu et al. (*37*), who found that five different tri- and pentavalent As compounds were positive in the *rec* assay with *B. subtilis*. On the other hand, arsenite and arsenate were not mutagens in Salmonella (*38*). Using a spot test, Rossman et al. (*39*) found that arsenite was not mutagenic in a number of *E. coli* strains with different repair capabilities and that arsenite was not mutagenic to *E. coli* wild type in treat and plate test and fluctuation test. Arsenite was not mutagenic in Chinese hamster V79-E cells (*39*) and NaHAsO$_4$ was not mutagenic in the L5178Y/TK assay (*40*). Na$_2$HAsO$_4$ induced morphological transformation of HEC *in vitro* (*27*). Arsenic compounds induce chromosome aberations in human lymphocytes after *in vivo* and *in vitro* exposure (*42-44*).

Burgdorff (*20*) found increased SCE in lymphocytes from six arsenic-treated persons, while Nordenson et al. (*45*) found the same SCE rate in eight arsenic-treated persons as in controls. Zanzoni et al. (*46*) found that *in vitro* exposure to arsenic increased SCE in human lymphocytes. Paton and Allison (*47*) found that both in a human diploid cell line and in human lymphocytes *in vitro* arsenite was a much more powerful inducer of chromosome aberrations than arsenate, which only increased the aberration rate slightly over the control value. This is in agreement with the findings reported here concerning effects of arsenic compounds on SCE and suggests that trivalent arsenic is closer to an "ultimate carcinogenic metabolite" than pentavalent arsenic.

From their negative mutagenesis studies in bacteria and Chinese hamster cells, Rossman et al. (*39*) suggested that arsenite might rather be a cocarcinogen than a carcinogen. This possibility has been investigated experimentally in mice (*48-51*) without any evidence of a cocarcinogenic effect of arsenic appearing in the results. The present author finds it more likely that differences in intracellular metabolism between different cell systems are responsible for the differences in effect on SCE observed in this study, and may explain both the negative results reported (*39,40*) and the striking difference between the carcinogenicity of arsenic compounds in man and experimental animals.

Beryllium. Be(II) was a weak inducer of increased SCE in P388D$_1$, but did not affect SCE in lymphocytes from two donors (Table 4). Epidemiological studies suggest that beryllium is a human carcinogen, but IARC (*33*) does not find the human evidence conclusive. Insoluble Be compounds have been demonstrated to induce osteosarcomas in experimental animals. Both soluble and insoluble Be compounds induce lung cancer after inhalation (*33,52*). Be compounds are not mutagenic in Salmonella (*53,54*), while the rec assay with *B. subtilis* yielded contradictory results (*36,37*). BeSO$_4$ is mutagenic to CHO-cells *in vitro* (*55*), increase misincorporation in cell-free DNA synthesis *in vitro* (*56*), induce morphological transformation of HEC *in vitro* (*41*) and enhance viral-induced transformation of HEC *in vitro* (*27*). In one study, millimolar concentrations of Be^{2+} induced chromosome aberrations in mammalian cells *in vitro* (*57*) while micromolar concentrations gave negative results in another (*47*).

Cadmium. Cd(II) was a weak inducer of SCE in P388D$_1$ in three experiments. The data of one are shown (Table 5). Cd(II) increased SCE in lymphocytes from four donors, but did not affect SCE in lymphocytes from three other donors. Andersen et al. (*26*) and Deaver and Campbell (*58*) found that Cd^{2+} did not increase SCE in Chinese hamster

Table 4. Effect of BeSO$_4$ on SCE.

Cell type	Compound	M	Increase in SCE compared with control, %	p
P388D$_1$	BeSO$_4$	10^{-4}	17	ns
–	–	10^{-5}	18	ns
–	–	10^{-6}	4	ns
HL[a]	–	10^{-4}	9.4	ns
–	–	10^{-5}	–2.5	ns
–	–	10^{-6}	–3.7	ns

[a]48-hr exposure; 10 μg/mL BrdU.

Table 5. Effect of CdCl$_2$ on SCE.

Cell type	Compound	M	Increase in SCE compared with control, %	p
P388D$_1$	CdCl$_2$	10^{-5}	30	< 0.025
–	–	10^{-6}	3.8	ns
–	–	10^{-7}	11	ns
HL[a,b]	–	10^{-5}	33	< 0.0025
–	–	10^{-6}	17	< 0.05
–	–	10^{-7}	9	ns
HL[a,c]	–	10^{-5}	–0.1	ns
–	–	10^{-6}	9	ns
–	–	10^{-7}	4	ns

[a]Two different donors were used.
[b]20 μg/mL BrdU; 72-hr exposure to CdCl$_2$.
[c]4 μg/mL BrdU; 48-hr exposure to CdCl$_2$.

V79-E cells and CHO cells. The importance of the BrdU concentration and CdCl$_2$ exposure time in relation to effect of Cd(II) on SCE in human lymphocytes is not clear from the data obtained so far, but in three of three experiments using 20 μg/mL, BrdU increased SCE was observed, while in one of three experiments using 10 μg/mL BrdU increased SCE was observed. One experiment with 4 μg/mL BrdU did not show increased SCE, suggesting that Cd^{2+} and 5BU may act synergistically on SCE. (Data from experiments with 20 and 4 μg/mL BrdU in human lymphocytes are shown).

Genetic factors could also be of importance for the differences observed between different donors as well as dietary and occupational Cd exposure due to a possible effect on metallothionein (MT) induction. The significance of MT for Cd metabolism is widely recognized (*59*) but so far the possible role of MT in the genotoxicity of Cd has only been investigated in one study (*58*), showing that precultivation of CHO cells in a low Cd^{2+} concentration protects the cells from induction of chromosome aberrations by a higher dose, capable of inducing chromosome aberrations in cells not previously exposed to Cd^{2+}. This induced resistance is correlated with increased MT synthesis (*60*).

A few epidemiological investigations report increased frequencies of respiratory and prostatic cancer among workers occupationally exposed to high Cd doses (*61,62*). Although the epidemiological evidence may be inconclusive, the most reasonable interpretation of the available data is that cadmium should be regarded as a human carcinogen. Parenteral injections of soluble and insoluble cadmium compounds have induced local sarcomas and remote tumors of the testes in experimental animals (*62,63*).

Cd(II) has given both negative (*36,64*) and positive (*37*) results in mutagenicity tests in bacteria.

Cd(II) is mutagenic in mammalian cells (*40,55*), increases misincorporation in cell-free DNA synthesis *in vitro* (*56*), induces morphological transformation of HEC *in vitro* (*41*) and enhances viral-induced transformation of HEC (*27*). Reports about the effect of human Cd exposure (dietary or occupationally) on the frequency of chromosome aberrations in peripheral lymphocytes are contradictory, since positive (*65*) and negative (*66,67*) results have been published. Bauchinger et al. (*68*) and Deknudt and Leonard (*69*) found increased frequencies of chromosome aberrations in lymphocytes from Cd-exposed workers, but these workers had also been exposed to other metals (Pb, Zn).

Shiraishi et al. (*70*) found that *in vitro* exposure of human lymphocytes to CdS induced chromosome aberrations, but others (*47,71*) did not find increased rates of chromosome aberrations in different mammalian cell types after continuous exposure to various Cd compounds. Deaven and Campbell (*58*) found that the extent of induction of chromosome aberrations in CHO cells by Cd^{2+} was dependent on the source of serum in the medium and suggested that this might explain the discrepancies between earlier studies.

Röhr and Bauchinger (*72*) found that while continuous exposure of Chinese hamster cells to Cd(II) did not increase the rate of chromosome aberrations, 3 hr exposure to a dose which would be lethal during continuous exposure induced a high rate of chromosome aberrations in the following recovery period. Thus, the available data indicate that Cd has a weak chromosome-breaking capacity, but that synergism with other metals may increase the yield of chromosome aberrations after combined exposures.

Chromium. $K_2Cr_2O_7$ increased SCE in two experiments with $P388D_1$ cells and in lymphocytes from four donors, but the increase varied considerably between different donors (Table 6).

Aaseth et al. (*73*) found that Cr(VI) was reduced to Cr(III) *in vitro* by GSH in an enzyme-independent reaction. Erythrocytes incubated with chromium compounds *in vitro* took up large amounts of Cr(VI), but not Cr(III). The Cr(VI) uptake could be decreased by either reacting Cr(VI) with GSH before addition to erythrocytes, or by depleting the erythrocytes from GSH by incubation with diethyl maleate. Thus, when whole blood cultures from different donors are exposed *in vitro* to Cr(VI), large variations in the effective dose to lymphocytes may be expected because of competition between lymphocyte and erythrocyte Cr(VI) uptake, and reduction in the medium of Cr(VI) by GSH due to even slight leakage or hemolysis of erythrocytes.

In two experiments with $P388D_1$ cells and with lymphocytes from one donor, $Cr(CH_3COO)_3$ increased SCE, while $Cr(CH_3COO)_3$ only slightly increased SCE in three other donors. Cr_2O_3, which is insoluble, did not increase SCE in two experiments with $P388D_1$. The uptake of Cr_2O_3 into $P388D_1$ cells was confirmed by phase-contrast microscopy. Representative data from the experiments are shown in Table 6. Cr(III) increased SCE approximately 10 times as effectively in $P388D_1$ as in human lymphocytes from one donor, and comparison with other donors revealed even larger differences (data not shown).

The carcinogenicity of chromium compounds in humans and experimental animals has been reviewed recently by IARC (*33*) and Norseth (*74*). In several studies, trivalent chromium compounds did not induce mutations in bacteria (*36,64*) and mammalian cells (*75*) and did not induce chromosome aberrations (*71,76*) and SCE (*77*), while hexavalent chromium compounds are strong mutagens in bacteria (*36,38, 64,78-80*), yeast (*81*) and mammalian cells (*75*), and induce chromosome aberrations (*71,76,82,83*), increased SCE (*77,82,83*) in mammalian cells *in vitro* and morphological transformation in BHK21 cells (*84*) and HEC (*41,76*). Nakamuro et al. (*85*) found that although hexavalent chromium compounds were much more powerful inducers of mutations in bacteria and of chromosome aberrations in mammalian cells than trivalent chromium compounds, the trivalent compounds did have a positive effect in both systems. Whiting et al. (*86*) found that while chromate induced unscheduled DNA synthesis (UDS) in human fibroblasts, Cr(III) was inactive. In contrast to results obtained in cell systems, Cr(III) was

Table 6. Effect of chromium compounds on SCE.

Cell type	Compound	M	Increase in SCE compared with control, %	p
$P388D_1$	$K_2Cr_2O_7$	10^{-6}	163	< 0.0005
–	–	10^{-7}	19	ns
–	–	10^{-8}	9	ns
HL^a	–	10^{-5}	62	< 0.0005
–	–	10^{-6}	37	< 0.0005
–	–	10^{-7}	31	< 0.0005
$P388D_1$	$Cr(CH_3COO)_3$	10^{-4}	151	< 0.0005
–	–	10^{-5}	41	< 0.0025
–	–	10^{-6}	6.5	ns
HL^a	–	10^{-3}	115	< 0.0005
–	–	10^{-4}	24	< 0.01
–	–	10^{-5}	5	ns
$P388D_1$	Cr_2O_3	10^{-3}	14	ns
–	–	10^{-4}	5.4	ns
–	–	10^{-5}	7.5	ns

[a]Two different donors were used; 48-hr exposure; 10 µg/mL BrdU.

more active than Cr(VI) in cell-free *in vitro* systems, inducing miscoding in DNA synthesis (56) and stable DNA-protein crosslinks in isolated nuclei and in solution (87).

The different results obtained with Cr(III) in the two cell systems used in this study may be explained by differences in cellular uptakes: while hexavalent chromium is taken up by cells via the anion transport system (88), the Cr(III) ion does not pass the cell membrane (89). After cellular uptake, intracellular Cr(VI) is reduced to Cr(III) either by reduced GSH or by NADH- or NADPH-dependent microsomal cytochrome P-450 catalysis (90). In macrophages, Cr(III) can be taken up by phagocytosis. Thus, the results presented here agree with the cited reports about negative effects of Cr(III) in whole cells and positive effects of Cr(III) in cell-free systems and suggest that Cr(III) is an ultimate carcinogenic form of chromium.

Cobalt. $CoCl_2$ increased SCE in P388D$_1$ cells and in lymphocytes from two donors (Table 7). There are no epidemiological studies available concerning the possible carcinogenic effect of occupational cobalt exposure of humans although heavy exposure has taken place. In animal experiments parenteral injections of insoluble cobalt compounds induced local sarcomas (91,92).

In the *rec* assay with *B. subtilis*, cobalt compounds gave both negative (36) and positive (37) results. Co(II) induces infidelity in DNA synthesis *in vitro* (56) and enhances viral-induced morphological transformation of HEC *in vitro* (27). Co(II) did not induce chromosome aberrations in human lymphocytes (47). Co(II) induces *petite* mutations in yeast (93,94).

Lead. $Pb(CH_3COO)_2$ increased SCE slightly in lymphocytes from two donors (Table 8). In a preliminary experiment some increase was seen after exposure of P388D$_1$ cells to Pb(II), but further experiments are needed to confirm this observation. IARC (33) reviewed the epidemiological data about cancer mortality among lead- and organolead-exposed workers and concluded that evaluation of

Table 7. Effect of $CoCl_2$ on SCE.

Cell type	Compound	M	Increase in SCE compared with control, %	p
P388D$_1$	$CoCl_2$	10^{-4}	70	< 0.0005
–	–	10^{-5}	16	ns
–	–	10^{-6}	0	ns
HL[a]	–	10^{-5}	23	< 0.01
–	–	10^{-6}	12	ns

[a]72-hr exposure; 20 μg/mL BrdU.

Table 8. Effect of $Pb(CH_3COO)_3$ on SCE.

Cell type	Compound	M	Increase in SCE compared with control, %	p
P388D$_1$	$Pb(CH_3COO)_3$	10^{-4}	9.9	ns
–	–	10^{-5}	24	ns
–	–	10^{-6}	-4.3	ns
HL[a]	–	10^{-4}	24.3	< 0.05
–	–	10^{-5}	16.8	ns
–	–	10^{-6}	-1.1	ns

[a]72-hr exposure; 10 μg/mL BrdU.

the carcinogenicity of lead was impossible. In the absence of adequate human data, the IARC recommended that lead compounds be regarded as if they presented a carcinogenic risk to humans. Dietary and parenteral administrations of soluble lead compounds to experimental animals have induced renal cancers in several studies (33).

Lead compounds are not mutagenic in bacteria (36,37,53,54,80). Hsie et al. (55) found that Pb(II) was mutagenic in CHO cells, while Amacher and Paillet (40) found that Pb(II) was not mutagenic in L51784/TK cells. Pb(II) increased infidelity of DNA synthesis *in vitro* (56), induced morphological transformation of HEC *in vitro* (95) and enhanced viral-induced transformation of HEC *in vitro* (27). Beek and Obe (96) found that *in vitro* exposure to Pb(II) increased the frequency of chromosome aberrations in human lymphocytes, but in several other *in vitro* studies Pb(II) did not induce chromosome aberrations in human lymphocytes (47,82,97,98) and Chinese hamster cells (99). Human lead exposure was found to increase the frequency of chromosome aberrations in periferal blood lymphocytes in nine studies, while in six other studies, no increase was found (33). Combined exposures took place in several of these studies, and a causative role of Pb in induction of chromosome aberrations cannot be certain. Beek and Obe (100) found that *in vitro* exposure to Pb(II) did not increase SCE in human lymphocytes, while Wulf (101) found that *in vitro* exposure to Pb(II) increased SCE in human lymphocytes from four donors of five tested. Grandjean et al. (18) found that in a group of long-term lead-exposed workers the SCE rates in peripheral lymphocytes increased significantly with increased blood concentrations of zinc protoporphyrin. SCE rates decreased significantly in relation to decreased lead exposure parameters during a period without lead exposure.

Manganese. Mn(II) increased SCE in P388D$_1$ cells and in lymphocytes from two of three donors tested (data shown from one experiment in each cell

type) (Table 9). There is no epidemiological evidence that Mn is a human carcinogen. $MnCl_2$ induced lymphosarcomas in mice (102). Furst (103) found that intramuscular administration of manganous acetylacetonate to rats induced local fibrosarcomas, whereas manganese powder and manganese dioxide did not induce tumors.

Mn(II) is reported to be mutagenic in bacteria (104), phages (105), yeast (106,107) and mammalian cells (55). Mn compounds gave contradictory results in the rec assay (36,37). Mn(II) induced misincorporation in DNA synthesis in vitro (56), enhanced viral-induced transformation of HEC in vitro (27) and induced chromosome aberrations in mammalian cells in vitro (71,108). Mn(II) enhanced the chromosome damaging effect of ascorbate (108) and isoniazid (109) and enhanced UDS induced by isoniazid and other hydrazines (110). In these studies H_2O_2 was probably the active metabolite producing free radicals responsible for the observed DNA damage, since addition of catalase or GSH reduced the enhancing effect of Mn (109,110). Whether the effect of Mn in the present study might be due to catalysis of redox processes involving medium components creating genotoxic metabolites or whether the Mn(II) ion is active per se cannot be determined from the results.

Mercury. CH_3HgCl, but not $HgCl_2$, increased SCE in P388D$_1$ in preliminary experiments. In human lymphocytes from two donors, $HgCl_2$ increased SCE, whereas no effect was observed in lymphocytes from two other donors (Table 10). CH_3HgCl increased slightly SCE in lymphocytes from three different donors. There are no data (clinical, epidemiological or from animal experiments) that indicate any carcinogenic effects of Hg compounds. The induction of peritoneal sarcomas after IP administration of mercury droplets to rats observed by Druckrey et al. (111) may be more related to foreign body carcinogenesis than to an effect of mercury (112,113). Hg compounds tested for mutagenicity in bacteria gave contradictory results in the

rec assay (36,37) and negative results in reversion assay (64). $HgCl_2$ enhanced viral-induced transformation of HEC in vitro (27). Organic mercury compounds are strong inducers of chromosome breaks in plant cells (114), and occupational and dietary exposure to organic mercury compounds increased the frequency of chromosome aberrations in exposed individuals (115-117). Organic mercury compounds are weak mutagens in vivo in Drosophila (114,118) and in Chinese hamster cells in vitro (119). Other genetic effects of mercury (spindle effects and in vitro induction of aneuploidy) have been reviewed by Ramel (114).

Nickel. In a preliminary experiment with 388D$_1$ cells, a weak effect on SCE was noted, but a clear dose-response was not achieved. In human lymphocytes from one donor, $NiSO_4$ increased SCE slightly but significantly, while $NiSO_4$ had no effect on SCE from another donor (data not shown).

The carcinogenicity of nickel in humans has been demonstrated in several epidemiological investigations. In animal experiments, parenteral administration of a number of slightly soluble Ni compounds induced local sarcomas (reviewed by 63).

Nickel compounds are not mutagenic in bacteria (36,37,120). Ni(II) increased misincorporation in DNA synthesis in vitro (56), induced mutations in mammalian cells in vitro (40,55), and morphological transformation of HEC in vitro (41), enhanced viral induced transformation of HEC in vitro (27) and induced chromosome aberrations in mammalian cells in vitro (121). Two groups of workers in a nickel

Table 9. Effect of MnSO₄ on SCE.

Cell type	Compound	M	Increase in SCE compared with control, %	p
P388D$_1$	$MnSO_4$	10^{-4}	45	< 0.005
–	–	10^{-5}	39	< 0.0125
–	–	10^{-6}	30	< 0.05
HL^a	–	10^{-7}	13	ns
–	–	10^{-5}	20	< 0.025
–	–	10^{-6}	9	ns

[a]72-hr exposure; 20 μg/mL BrdU.

Table 10. Effect of HgCl₂ and CH₃HgCl on SCE.

Cell type	Compound	M	Increase in SCE compared with control, %	p
P388D$_1$	$HgCl_2$	10^{-5}	10	ns
–	–	10^{-6}	13	ns
–	–	10^{-7}	–12	ns
–	CH_3HgCl	10^{-5}	94	< 0.0005
–	–	10^{-6}	14	ns
–	–	10^{-7}	2.8	ns
HL^{a,b}	$HgCl_2$	10^{-5}	46[c]	< 0.0005
HL^{a,d}	CH_3HgCl	10^{-5}	26[c]	< 0.0125
HL^{a,d}	$HgCl_2$	10^{-5}	8.7[c]	ns
–	–	10^{-5}	6.9[e]	ns
–	–	10^{-5}	5.3[f]	ns
–	CH_3HgCl	10^{-5}	20.3[c]	< 0.025
–	–	10^{-5}	18.7[e]	ns
–	–	10^{-5}	23.3[f]	< 0.025

[a]Three different donors were used.
[b]20 μg/mL BrdU.
[c]72-hr exposure.
[d]10 μg/mL BrdU.
[e]48-hr exposure.
[f]24-hr exposure.

refinery exposed mainly to insoluble (NiO, Ni_3S_2) or mainly to soluble ($NiCl_2$, $NiSO_4$) nickel compounds had increased rates of chromosome aberrations but not SCE in peripheral lymphocytes compared with a control group (*122*).

Effects of Organic Carcinogens on SCE in P388D₁

To investigate the metabolic capacity of the cell line $P388D_1$ for latent organic carcinogens, the effect of *in vitro* exposure of $P388D_1$ cells to BP and DMN on SCE was measured (Table 11). As a positive control, thio-TEPA, a direct acting alkylating agent, was included. Thio-TEPA induced a dose-dependent increase in SCE. Both BP and DMN were tested at large concentration ranges, including toxic concentrations. The incubation period with these compounds was 48 hr, giving sufficient time for induction of microsomal mixed-function oxygenases and activation of the carcinogens. Since neither of the compounds increased SCE, it is likely that the $P388D_1$ cell line has lost capacity for metabolization of latent carcinogens, as have other transformed cell lines. This decreases the utility of this cell line for evaluation of mutagenic/carcinogenic properties of complex compounds, since only the effect of direct acting organic substances will be monitored.

Effects of Coal Combustion Products on SCE in P388D₁

Samples of fly ash from the precipitators of four different coal-fired plants, a flue gas desulfurization sample and emission samples collected at conditions comparable to those in the flue gas plume during cooling have been tested for effects on SCE in $P388D_1$. The sampler is briefly described in the "Materials and Methods" section. Details regarding plants and samples are summarized in Table 1.

All samples tested were toxic at high concentrations. The most toxic among the flue gas cleaning samples was the flue gas desulfurization sample (B-2).

The emission samples were more toxic than the precipitated samples, which allowed SCE determination after exposure of cultures to 10 mg (Table 12). The emission sample with particle size > 3 μm, collected downstream from the dynamic precipitators at a coal-fired plant (sample D-3), was the least toxic, only slightly more toxic than precipitated fly ash. This sample allowed SCE scoring after exposure to 5 mg. The smaller particles of the same emission sample (sample D-2) were more toxic, allowing SCE scoring after exposure to 2–5 mg,

Table 11. Effect of organic carcinogens on SCE in P388D₁.

Compound	M	Increase in SCE compared with control, %	p
Thio-TEPA	10^{-5}	243	< 0.0005
–	10^{-6}	47	< 0.0005
–	10^{-7}	19	ns
BP	2×10^{-5}	14	ns
–	2×10^{-6}	16	ns
–	2×10^{-7}	27	< 0. 025
–	2×10^{-8}	12	ns
–	2×10^{-9}	11	ns
DMN	1.35×10^{-2}	12	ns
–	1.35×10^{-3}	-9.7	ns
–	1.35×10^{-4}	-5.8	ns
–	1.35×10^{-5}	-14.5	ns

Table 12. Effect of fly ash on SCE in P388D₁.

Sample	Concn, mg/culture	Increase in SCE compared with control, %	p
C	10	14	ns
	1	9	ns
	0.1	15	ns
A	10	20	ns
	1	9	ns
	0.1	15	ns
B-1	10	25	< 0.05
	1	18	ns
	0.1	-4	ns
B-2	10	18	ns
	1	7	ns
	0.1	7	ns
D-1	10	7	ns
	1	6	ns
	0.1	-12	ns

while the most toxic sample was that emitted from an oil-fired plant without flue gas cleaning (sample E), allowing SCE scoring after exposure to 1–2 mg. None of the precipitated fly ash samples had a strong effect on SCE, but one of the samples (B-1) slightly increased SCE. This sample was reinvestigated in two experiments (Tables 13 and 14). A 400-mg portion of precipitated fly ash, sample B-1, was incubated with 10 ml MEM + 10% fetal calf serum with occasional shaking at 37°C for 1 hr or 24 hr to extract soluble components. After centrifugation at 3000 rpm for 5 min the supernatants were aspirated and the precipitates resuspended in 10 mL medium with serum. In each experiment three series of cultures were exposed 48 hr to 5BU and various amounts of unextracted fly ash, extracted fly ash and the soluble fraction. Both after 1 hr and 24 hr extraction, the extracted fly ash remained toxic: 20 mg unextracted and 1 hr extracted fly ash

induced too high toxicity to allow SCE determination, although a few first mitoses were still present. A 10 mg portion of 1-hr extracted or 24-hr extracted fly ash induced almost the same toxicity as did 10 mg unextracted fly ash. The soluble fraction was added to cultures in different amounts and the dose is stated in the tables as milligrams fly ash extracted. Addition of 1 hr and 24 hr extracts corresponding to 20 mg fly ash did not induce toxicity in the cultures. Even an amount of 24 hr extract corresponding to 80 mg fly ash did not kill the cultures, but induced mild toxicity allowing SCE scoring.

This does not necessarily mean that the soluble fraction is less toxic than the insoluble fraction, since these different exposure situations cannot be directly compared. Addition of insoluble material which precipitates in close contact with the macrophages yields a much larger effective dose due to phagocytosis than addition of the same amount of soluble material, which is diluted into the total amount of medium. But that a significant amount of toxic material remains in the fly ash even after 24 hr extraction was demonstrated by the high toxicity of the extracted fly ash.

In both these experiments the unextracted fly ash induced a dose-dependent increase in SCE. Fly ash extracted for 1 hr and 24 hr increased SCE slightly at high doses, but less than unextracted fly ash, demonstrating that some of the compounds in fly ash causing the increase in SCE had been extracted.

In agreement with this finding, both 1-hr and 24-hr extracts containing the soluble components of fly ash induced a dose-dependent increase in SCE. The highest dose of 24-hr extract tested induced a larger increase in SCE than the highest dose of unextracted fly ash, most likely due to separation of

Table 13. Effect of fly ash, 1-hr extracted fly ash and 1-hr extract from fly ash on SCE in P388D$_1$, sample B-1.

Compound	Concn, mg/culture	Increase in SCE compared with control, %	p
Fly ash	10	44	< 0.0025
	5	27	< 0.0025
	2	24	< 0.005
	1	17	< 0.05
Extracted fly ash	10	43	< 0.0025
	5	10	ns
	2	15	ns
	1	9	ns
Extract corresponding to	20	27	< 0.01
	10	22	< 0.025
	5	3	ns
	2	11	ns
	1	–2	ns

Table 14. Effects of fly ash, 24-hr extracted fly ash and 24-hr extract on SCE in P388D$_1$, sample B-1.

Compound	Concn, mg/culture	Increase in SCE compared with control, %	p
Fly ash	10	65	< 0.0005
	5	47	< 0.0005
	2	30	< 0.025
	1	22	< 0.05
Extracted fly ash	10	22	< 0.05
	5	23	< 0.05
	2	1	ns
	1	–4	ns
Extract corresponding to	80	92	< 0.0005
	40	53	< 0.0005
	20	26	< 0.05
	10	6	ns
	5	15	ns

slightly soluble toxic components remaining in the extracted fly ash from (possibly) less toxic soluble components present in the extract.

The results demonstrate that one of the tested samples of fly ash from precipitators is highly toxic and likely to be mutagenic. The extraction medium used to extract this sample mimic the conditions at the deposition target after inhalation of fly ash, and if fly ash is inhaled, the lung tissue will be exposed to a dose of compounds capable of increasing SCE, an indication of mutagenicity. This sample of fly ash was collected at the electrostatic precipitator after a cyclone. Thus, the positive result may be due to enrichment of the precipitated sample with small particles, since most of the coarse material is precipitated at the cyclone. If this plant had only been equipped with a cyclone, large amounts of material likely to be mutagenic would be emitted to the environment.

The particles that do escape the stack despite attempts to clean the smoke by different kinds of precipitators have different properties than the samples of precipitated fly ash. Due to condensation of organic (e.g., PAH and organic nitro compounds) and inorganic (e.g., Cd) compounds around small particles not retained, a new kind of particle is generated when the smoke leaves the stack. The effects of such emission samples on SCE are summarized in Table 15. The sample collected at an oil-fired plant without flue gas cleaning (sample E) did not increase SCE significantly. The same was found with sample D-3 (coal-fired, >3 μm), while sample D-2 (coal-fired, <3 μm) increased SCE in three independent experiments (Table 15).

Experiments with extraction for 24 hr, as those described above, were performed with the three emission samples (Table 16). The extract from sam-

Table 15. Effects of emission samples on SCE in P388D₁.

Sample	mg/culture	Expt. 1		Expt. 2		Expt. 3	
		Increase, %[a]	p	Increase, %[a]	p	Increase, %[a]	p
E	20	mi[b]	–	nt[c]	—	nt	–
	10	mi	–	nt	—	nt	–
	5	mi	–	mi	—	nt	–
	2	mi	–	7.2	ns	10.4	ns
	1	13.4	ns	8.1	ns	–1.8	ns
	0.5	nt	–	4.5	ns	–1.8	ns
	0.2	nt	–	–6.4	ns	–5.2	ns
	0.1	nt	–	–6.4	ns	–9.6	ns
D-2	20	mi	–	nt	—	nt	–
	10	mi	–	nt	—	nt	–
	5	mi	–	41.4	<0.0005	mi	–
	2	16.5	ns	13.5	ns	32.2	<0.005
	1	–4.8	ns	3.6	ns	22.6	<0.025
	0.5	nt	–	–10.8	ns	6.1	ns
	0.2	nt	–	5.4	ns	2.6	ns
	0.1	nt	–	4.5	ns	nt	–
D-3	20	mi	–				
	10	mi	–				
	5	3.1	ns				
	2	–11.1	ns				
	1	–3.9	ns				

[a]Increase in SCE compared with control, %.
[b]mi = mitotic inhibition.
[c]nt = not tested.

Table 16. Effects of extracts of emission samples on SCE in P388D₁.

Sample	Extract corresponding to, mg/culture	Increase in SCE compared with control, %[a]	p
D-2	40	mi	–
	20	mi	–
	10	mi	–
	5	mi	–
	2	23.4	ns
	1	39.3	> 0.005
	0.5	47.7	> 0.0025
	0.2	6.5	ns
E	20	mi	–
	10	mi	–
	5	mi	–
	2	6.6	ns
	1	–3.8	ns
	0.5	8.5	ns
	0.2	6.6	ns
	0.1	0	ns
D-3	20	mi	–
	10	mi	–
	5	–9.2	ns
	2	13	ns
	1	2.6	ns
	0.5	14	ns

[a]mi = mitotic inhibition.

ple E was highly toxic and did not increase SCE. Extract from sample D-3 (>3 μm), was also toxic, and did not increase SCE. Extract from sample D-2 (<3 μm), which was the only sample increasing

SCE, did also increase SCE. This extract was highly toxic.

The importance of the collection temperature, the particle size and the collection method for the mutagenicity of emission samples from coal-fired plants has been investigated in several studies. A review of work performed up to 1980 was published by Chrisp and Fisher (*123*). In most studies electrostatic precipitator-collected coal fly ash was not mutagenic. Condensation of organic mutagens around particles seems to take place when the flue gas is cooled to below 100°C. Chemical analysis of size-fractionated emission samples showed preferential accumulation of As, Cd and Ni on small particles. The mutagenicity of emission samples is inversely related to the particle size. Prolonged exposure of collected material to flue gas may induce chemical reactions creating mutagens. The major fraction of the mutagenic activity of coal fly ash is due to direct-acting mutagens which are soluble in serum, but not in phosphate-buffered saline (*124*). The results obtained in this study of four precipitated fly ash samples and two emission samples from coal-fired plants agree with these earlier studies.

The use of an established macrophage cell line allowed the direct assessment of possible mutagenic (SCE-increasing) activities of particulate emissions. It was possible to evaluate and compare the effects of particles, extracted particles and extract in the same cell system. The value of this cell line is

reduced both by inherent toxicity, probably due to a virus, and by its limited metabolic capacity, which it shares with other established cell lines.

Boorman et al. (125,126) demonstrated a limited division capacity of PAM in vitro. Preliminary studies by this author indicate that cytogenetic studies can be performed in such cells. The use of PAM instead of P388D₁ cells would improve considerably the value of direct evaluation of mutagenic effects of particulate emissions in macrophages.

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